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14. ABSTRACT

Breast cancer is the most frequently diagnosed cancer in women with about 180,000 new cases reported each year. Early detection allows the greatest chance for successful therapies which include surgical procedures, irradiation, hormonal, and chemical intervention. Yet, these do not always achieve complete recovery, so our goal was to develop novel techniques that might identify markers that would allow us to construct metabolic maps in different types of breast cancers to predict efficacy of therapeutic treatment options. Using NMR, we have now demonstrated that hypoxic treatment of a basal B, triple negative breast cancer cell line increases substantially the flux of non-glycolytic product(s) into the TCA cycle which might increase the ability of cells to use oxidative phosphorylation for ATP production. An inhibitor of the pentose phosphate shunt significantly altered glucose metabolism suggesting that this pathway makes a major contribution to metabolic flux. Identifying the non-glycolytic products that support mitochondrial metabolism will be a major direction in our work, as this impacts our understanding of mitochondrial function in breast cancer.

15. SUBJECT TERMS
Metabolomics; Breast Cancer; Pentose Phosphate Shunt, Glucose Metabolism.

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Introduction:

Breast cancer is the most frequently diagnosed cancer in women in the United States, with over 190,000 new cases predicted for 2009 (American Cancer Society, Breast Cancer Facts and Figures). Breast cancer is the second leading cause of cancer death in women and during this year it is predicted that 40,470 women will die from the disease. This number is equivalent to one death every 13 minutes. Early cancer detection allows the greatest chance for successful therapy which includes surgical procedures, irradiation, hormonal, and chemical intervention. Despite recent advances in diagnosis and therapeutics, complete recovery is not always realized. Therefore, novel approaches towards predicting therapeutic success and/or outcome of breast cancer patients are sought. Most of these approaches require tissue derived from tumors to detect the expression of the "family" of genes or specific proteins. In our proposal, we planned to use nuclear magnetic resonance spectroscopy (NMR) to evaluate metabolic intermediates in human breast cancer cells (hBrC) by isotopomer analysis. Our goal was to construct metabolic maps (tumorigenic metabolomes) to identify unique metabolic programs that are necessary for the developing metastatic phenotype of hBrC.

Body:

Statement of Work: *Task 1*

Task 1 described our efforts to gain approval for animal usage which is planned for year three. We were successful in this, and received the third year renewal in January, 2011. This serves the last half of the third year, and the no cost extension for six months. Unfortunately, we felt is impossible to complete the animal experiments as the levels of metabolites did not warrant the sacrifice of animals.

Statement of Work: *Task 2* (months 1-15)

Task 2 described our approach to compare the contribution of glycolysis, the pentose phosphate shunt (PPS), and oxidative metabolism to glucose metabolism in hBrC using ^{13}C-labeled glucose (months 1 - 15). We have previously reported on our first series of experiments comparing the comparing the isotopomer pattern of glutamate in MCF10A cells (the controls) with T47D (a luminal, ER positive breast cancer cell line) and MDA-MB-231 cells (a basal B, triple negative breast cancer cell line). In these experiments we used a four hour pulse of [U-^{13}C]glucose which revealed that the anapleurotic reactions from glucose metabolism that lead into the TCA cycle were down-regulated in the breast cancer lines, relative to the control epithelial line. Another goal for Task 2 was to evaluate the effect of inhibition of the pentose phosphate shunt. We have now conducted a number of experiments using an inhibitor of 6 phosphogluconate dehydrogenase (6PG-DH), 6-aminonicotinamide (see Figure 1).

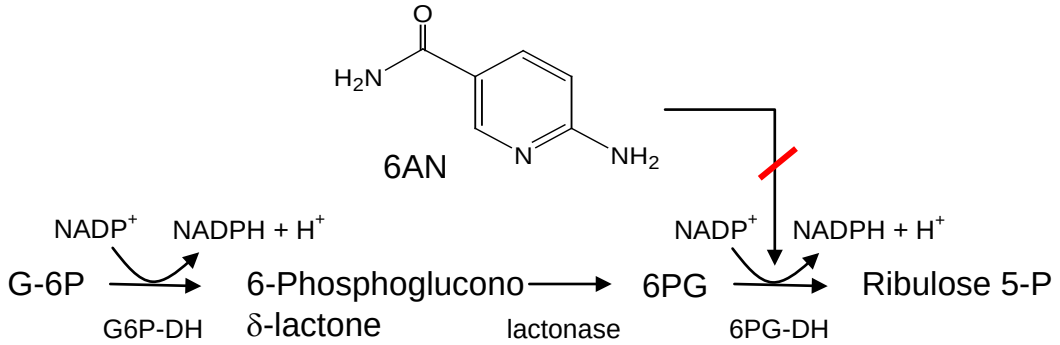


Figure 1 Illustration of initial enzymatic steps in the pentose phosphate shunt. G6P = glucose 6-phosphate; G6P-DH = glucose 6-phosphate dehydrogenase; 6PG = 6-phosphogluconate; 6PG-DH = 6-phosphogluconate dehydrogenase; 6AN = 6-aminonicotinamide.

These experiments were constructed so that hypoxia could be evaluated simultaneously with the effect of the inhibitor (the effect of hypoxia was technically Task 3). Thus, we changed our protocol to include a 4 hour exposure to normoxia or hypoxia in the presence or absence of 6AN

(200μM). This was followed by a 12 hour exposure to normoxia or hypoxia, in the presence or absence of 6AN, but now including 15mM [U-^{13}C]glucose (or 15mM normal isotopic glucose for protein evaluation). Figures 2 through 4 show the effect of 6AN on metabolic indices.

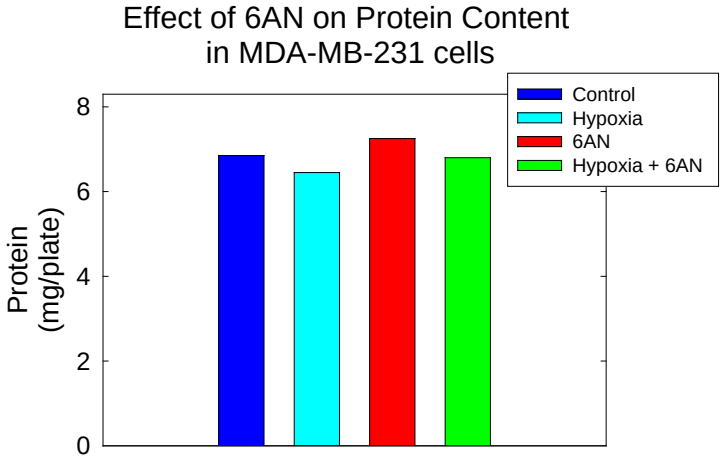


Figure 2 Effect of 6AN on protein content of MDA-MB-231 cells. MDA-MB-231 cells, on day three after plating, were exposed to normoxic or hypoxic conditions (1% oxygen) in the presence or absence of 200μM 6AN (total of 16 hours). Cells were washed with ice-cold PBS, and protein extracted for analysis using the Markwell technique [1] Data represent the average of two independent experiments. No statistical significance was observed between samples.

We show in Figure 2 that the protein content over the course of the experiment did not fluctuate in response to the conditions. This was an important point, as these data give us confidence that the same number of cells/equivalent protein is utilized in the NMR analysis.

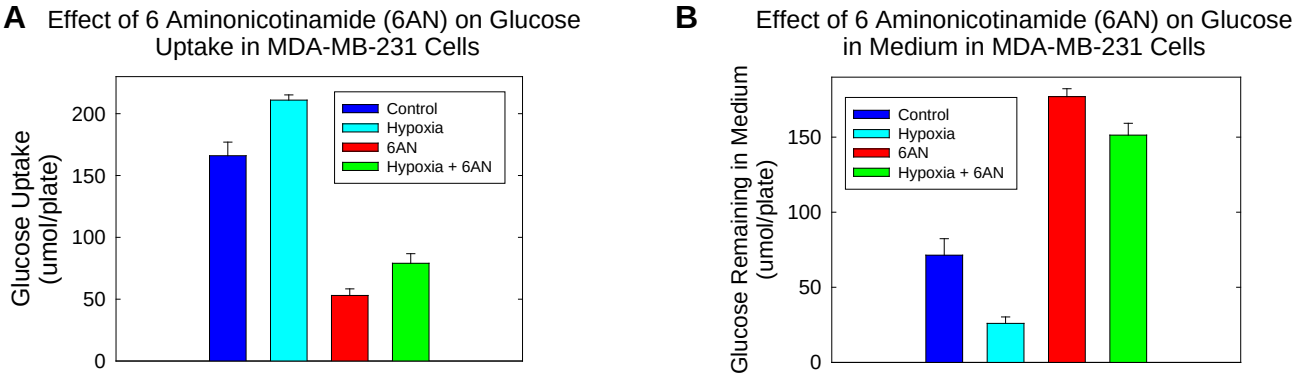


Figure 3 Effect of 6AN on glucose uptake in MDA-MB-231 cells. MDA-MB-231 cells were grown for three days at which point they were exposed to normoxic or hypoxic conditions in the presence or absence of 6AN for 4 hours. At that point, fresh medium containing 15mM [U-^{13}C]glucose or normal isotopic glucose with or without 6AN was added to the cells and incubation under normoxic or hypoxic conditions was continued. Data represent average uptake of glucose into cells (Panel A) or glucose loss from the medium (Panel B) in two independent sets of experiments.

Data in Figure 3 demonstrate several metabolic features. First, it is clear that hypoxia increases glucose uptake, Panel A (i.e., removal of glucose from the extracellular medium) compared to cells exposed to normoxia. Second, 6AN indirectly blocks glucose uptake which one might assume would slow glucose metabolism. This was further evaluated by measuring lactic acid output (Figure 4). Hypoxia increases lactic acid production, as expected. 6AN exposure significantly reduces lactate production under both control (normoxic) and hypoxic conditions. The striking effect of 6AN on glucose uptake and utilization was unexpected, although moderate inhibition was noted in perfused RIF-1 cells by Street et al. [2] This may mean that much of the glucose is normally metabolized through the pentose phosphate shunt. Alternatively, the accumulated 6 phosphogluconate, which is a potent inhibitor of the phosphoglucose isomerase reaction, acts to inhibit glycolysis by blocking the conversion of glucose 6-P to fructose 6-P.

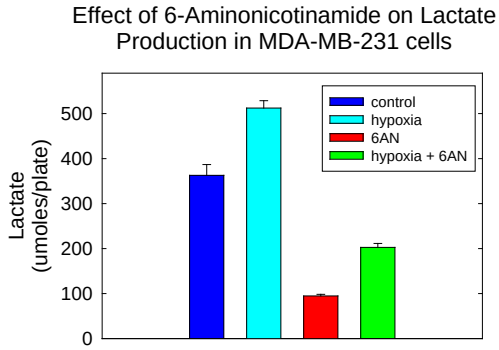


Figure 4 Effect of 6AN on lactic acid production in MDA-MB-231 cells. MDA-MB-231 cells, on day three after plating, were exposed to normoxic or hypoxic conditions (1% oxygen) for four hours in the presence or absence of 200μM 6AN. Fresh medium containing 15mM [U-^{13}C]glucose or ^{12}C-glucose with or without 6AN was added to the cells and incubation under normoxic or hypoxic conditions was continued (total of 16hours). Aliquots of the medium were taken and lactate determined using a spectrophotometric assay. Data represent the average of two independent sets of experiments.

The effect of 6AN exposure on the distribution of ^{13}C glucose carbons in various metabolites is shown in Figure 5. The obvious difference between the cells exposed to 6AN versus those that were not is the accumulation of gluconate (and the loss of metabolic intermediates). This is not unexpected given the data published by Street et al. [3].

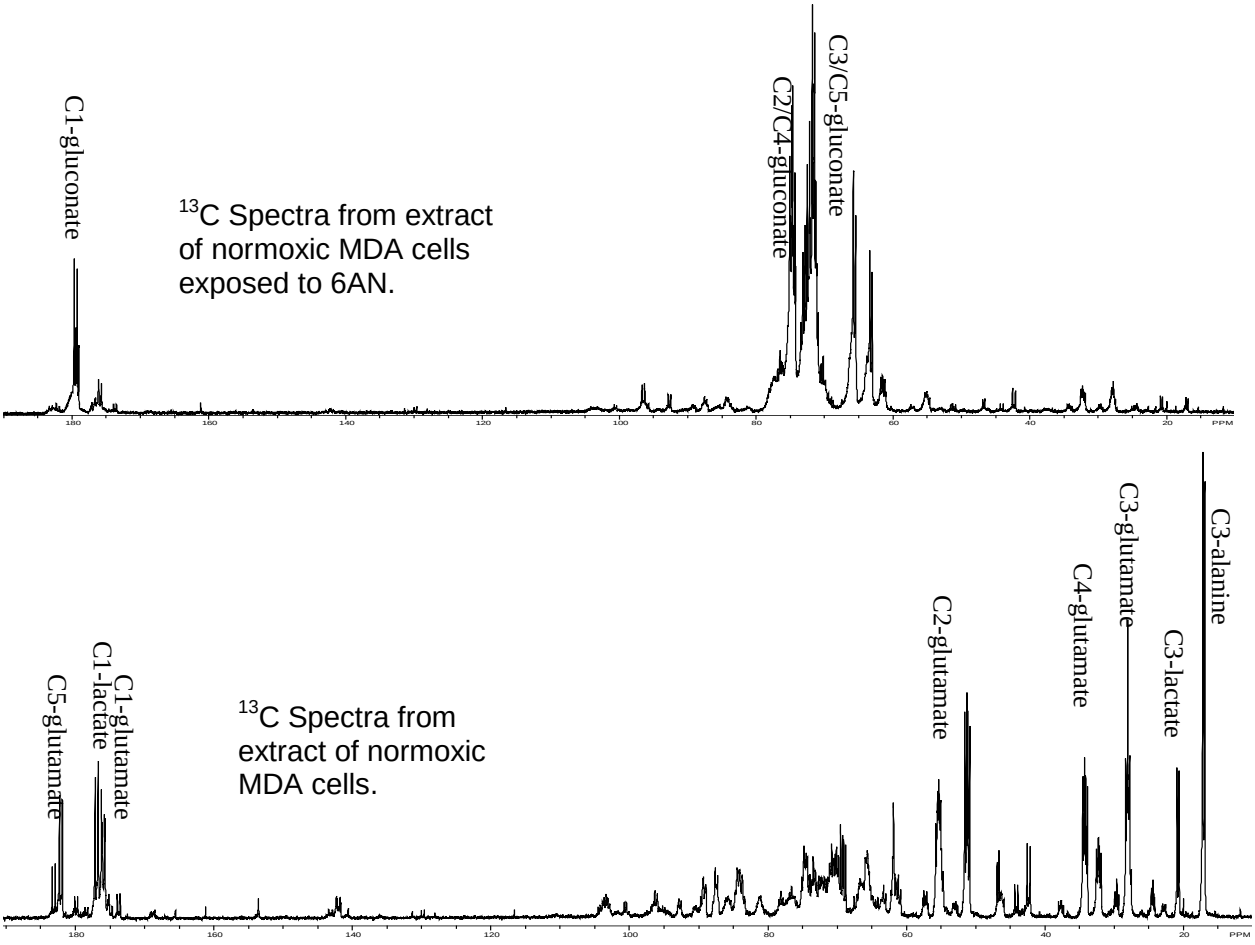


Figure 5. 6AN causes the accumulation of gluconates in normoxic MDA-MB-231 cells. See Figure 6 for experimental details.

Similar results were obtained with hypoxic cells as shown in Figure 6. The hypoxic controls (lower panel) show the accumulation of labeled amino acids and lactate, while the 6AN-treated hypoxic cells show elevated concentrations of gluconate, with reduced levels of amino acids and lactate.

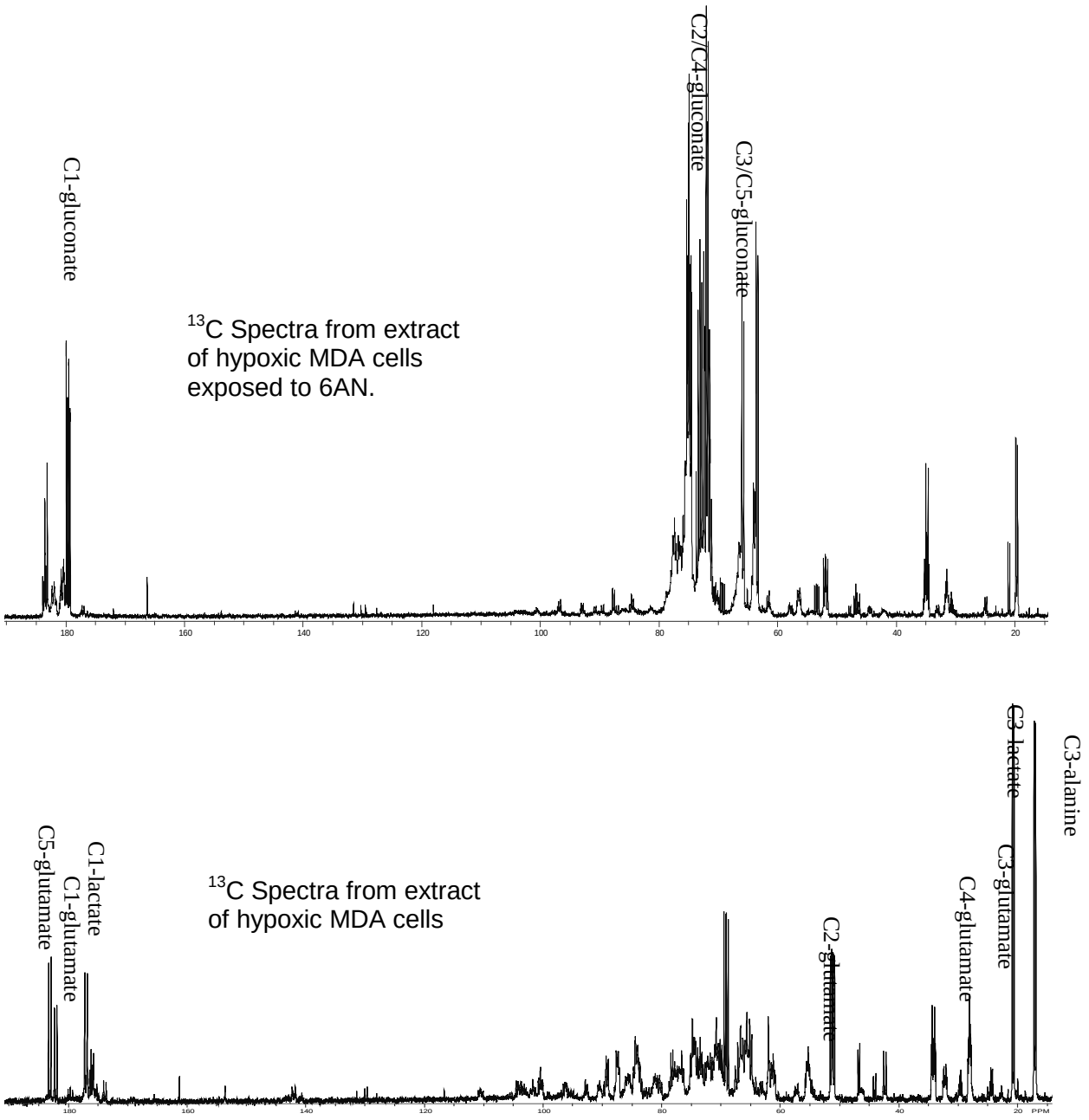


Figure 6 6AN increases gluconates in hypoxic MDA-MB-231 cells but preserves some metabolic activity. Cells from Figure 2 were washed in ice-cold saline to remove extracellular medium, extracted with ice-cold methanol, and scraped into separatory flasks. The methanolic cell slurry was diluted with an equal volume of chloroform and vigorously shaken. Ice-cold water was added and mixed to induce phase separation. The aqueous portion was collected and treated with Chelex-100, lyophilized, and resuspended in 0.5mL of D_2O. Proton-decoupled ^{13}C spectra were acquired at 500mHz in a Bruker instrument.

With the cells treated as described above, we were able to collect high quality spectra from their extracts. Shown in Figure 7 are the proton-decoupled ^{13}C-NMR spectra of the glutamate isotopomer patterns. Even to the untrained eye, clear differences in the peaks can be discerned. As illustrated in Figure 8, we modeled the glutamate data using tcaCALC (University of Texas, Southwestern). The model that best fits the normoxic data uses a single pyruvate pool with a single anapleurotic entrance (pyruvate carboxylase). The model that best fits the hypoxic data uses a single pyruvate pool with two anapleurotic entrances...one via pyruvate carboxylase and one via an unidentified source. For each step that is numbered,

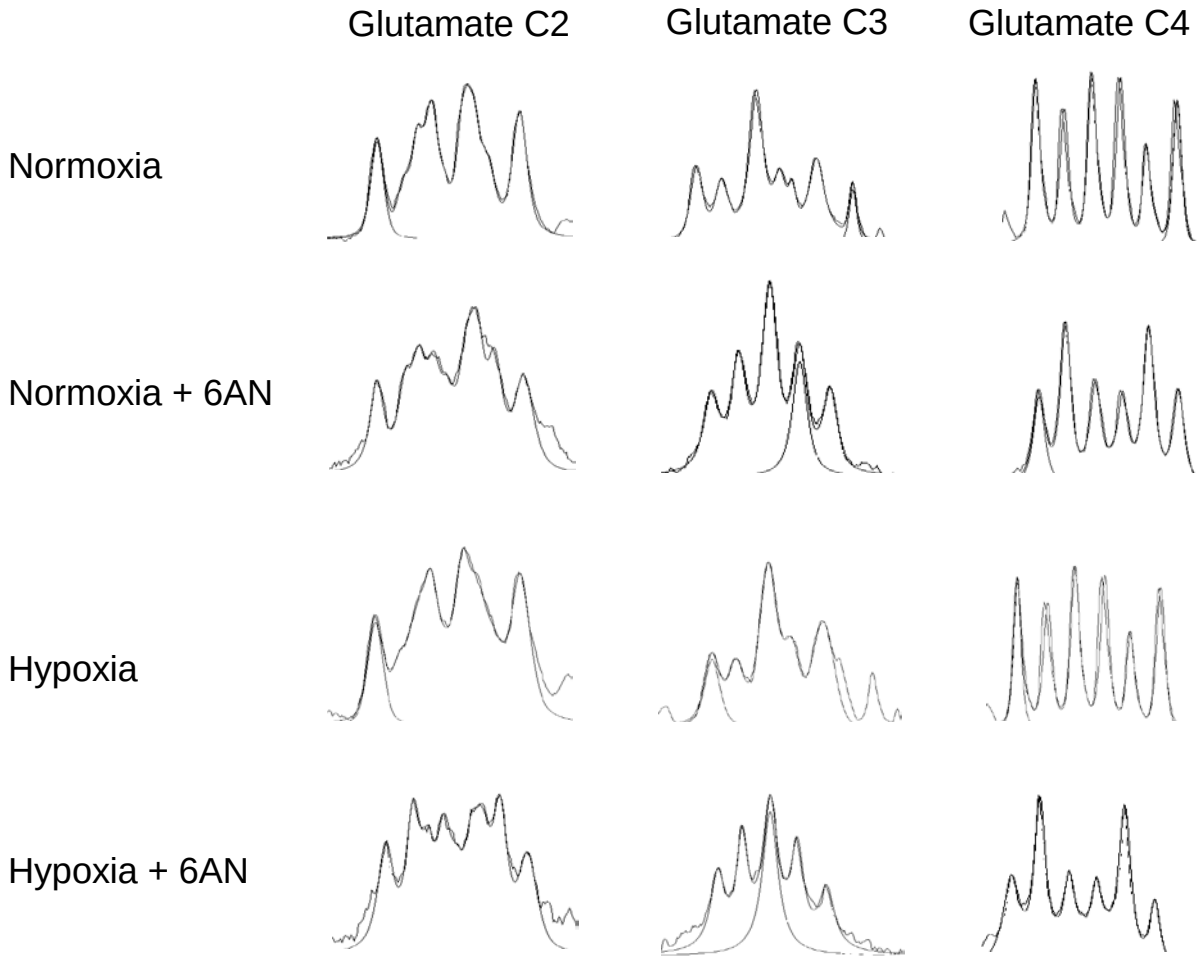
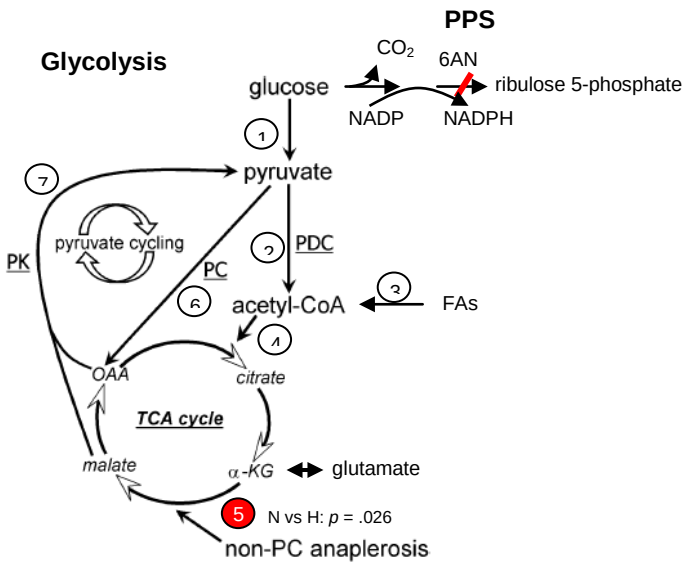


Figure 7 Isotopomer patterns of glutamate resonances in MDA-MB-231 cells in response to hypoxia and 6AN. Cells from Figure 2 were washed in ice-cold saline to remove extracellular medium, extracted with ice-cold methanol, and scraped into separatory flasks. The methanolic cell slurry was diluted with an equal volume of chloroform and vigorously shaken. Ice-cold water was added and mixed to induce phase separation. The aqueous portion was collected and treated with Chelex-100, lyophilized, and resuspended in 0.5mL of D_2O. Proton-decoupled ^{13}C spectra were acquired at 500mHz in a Bruker instrument.



	MDA-MB-231 cells			
	N	N + 6AN	H	H + 6AN
1	0.87	0.93	1.20	0.81
2	0.91	0.94	0.89	0.90
3	0.08	0.05	0.13	0.09
4	1.00	1.00	1.00	1.00
5	0.08	0.13	0.45	0.57
6	0.53	0.30	0.82	0.22
7	0.57	0.32	0.48	0.32

*

Figure 8 Isotopomer modeling using tcaCALC

the flux data for are shown in accompanying Table. Step 1 reflects the contribution to the pyruvate pool from ^{13}C-labeled glucose. These carbons can derive from glucose via glycolysis or the pentose phosphate shunt. Step 2 reflects the contribution of the pyruvate pool to the acetyl CoA pool via pyruvate dehydrogenase (in the illustration this is labeled as PDC (Pyruvate Dehydrogenase Complex). Step 3 reflects the contribution of fatty acid oxidation to the acetyl CoA pool (this pool will be unlabeled under our conditions). Step 4 reflects the contribution of acetyl CoA to the TCA cycle. By convention, this rate is defined at 1. All data are reported relative to this step. Step 5 reflects the contribution to the TCA cycle from intermediates other than pyruvate. This pool could reflect a number of amino acids (see below). Step 6 reflects the contribution to the TCA cycle from pyruvate via pyruvate carboxylase. Step 7 reflects pyruvate recycling via PEP carboxykinase and pyruvate kinase. The normoxic and hypoxic data were generated from triplicate sets of data while the 6AN data derive from duplicate sets of experiments. The most significant effect is the enhanced flux from an unidentified anapleurotic pool (step 5) in response to hypoxia, even in the presence of 6AN. While glutamate is often thought of as an anapleurotic substrate, studies which measure substrates across human tumors have failed to support net glutamate retention [4]. Rather, the branched chain amino acids and serine are retained by tumors indicating that they might serve as TCA substrates to maintain energy balance.

Finally, shown in Figures 9 and 10 are the ^{31}P spectra from the MDA-MB-231 cells. The data, below, suggest that hypoxia reduces the concentration of NTP (see red arrows, Figure 9) perhaps increasing the concentration of NDP (see blue arrows, Figure 9). The difference in signal to noise between the normoxic and hypoxic spectra underestimates the decrease in NTP in the hypoxic cells. The decrease in phosphocreatine (PCr) in hypoxic cells confirms the shift in energy charge. Cells treated with 6AN (Figure 10) show a significant increase in the 6-phosphogluconate peak, in fact “off the chart” (see green star). Furthermore, in cells treated with 6AN, phosphocholine (PCho) levels are reduced. Interestingly, both the normoxic and hypoxic cells show some NTP and NDP when exposed to 6AN, despite the strong block in glucose metabolism (Figures 3 and 4).

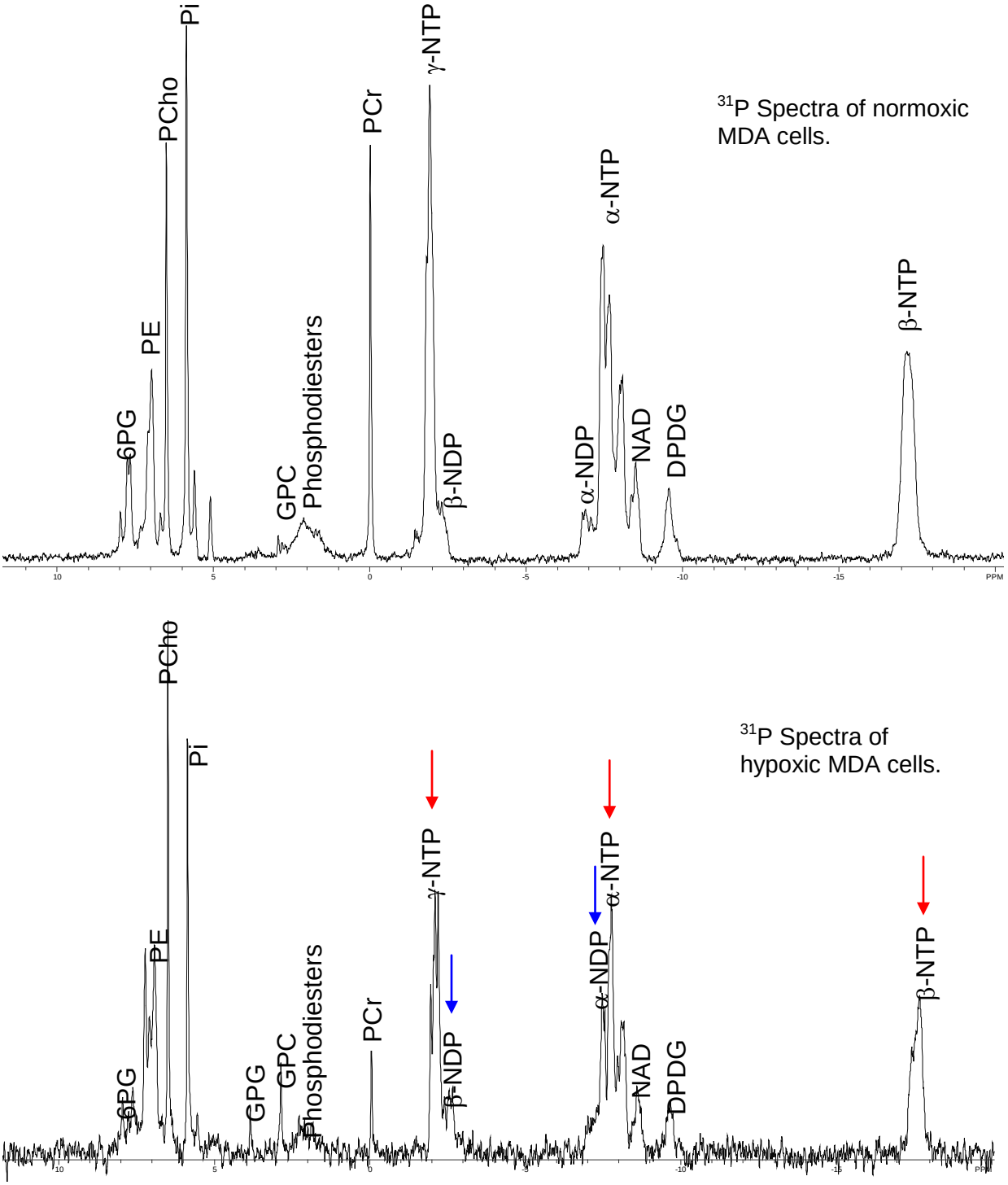


Figure 9 ^{31}P Spectra from extracts of MDA-MB-231 cells. Abbreviations: 6PG, 6-phosphogluconate; PE, phosphoethanolamine; PCho, phosphocholine; Pi, inorganic phosphate; GPG, glycerophosphoglycerol; GPC, glycerophosphocholine; PCr, phosphocreatine; X-NTP, nucleotide triphosphates. X-NDP, nucleotide diphosphates. NAD, nicotinamide adenine dinucleotide; DPDG, diphosphodiglycerides.

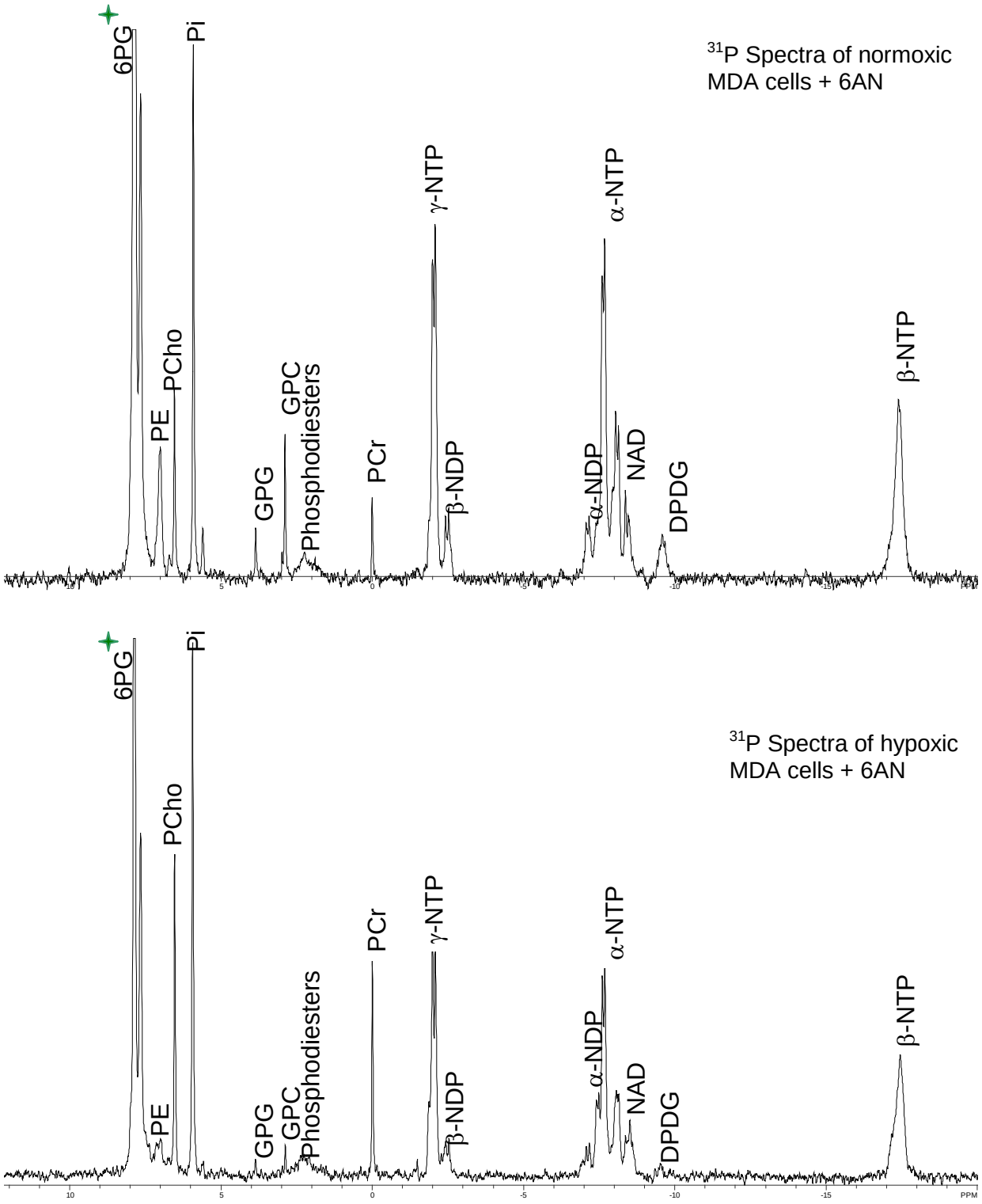


Figure 10 ^{31}P Spectra from extracts of MDA-MB-231 cells. Abbreviations: 6PG, 6-phosphogluconate; PE, phosphoethanolamine; PCho, phosphocholine; Pi, inorganic phosphate; GPG, glycerophosphoglycerol; GPC, glycerophosphocholine; PCr, phosphocreatine; X-NTP, nucleotide triphosphates. X-NDP, nucleotide diphosphates. NAD, nicotinamide adenine dinucleotide; DPDG, diphosphodiglycerides.

Unfinished goals: *Task 2c.* We also planned to evaluate an activator of pyruvate dehydrogenase (dichloroacetate, DCA). However, we have been unsuccessful in showing that DCA accelerates oxidative glucose metabolism. We have not pursued the analysis of metabolic products using ^{13}C-glucose.

Statement of work: *Task 3* (months 12-21)

Task 3 was to determine the effect of hypoxia on isotopomer patterns. We have made significant progress on this task as noted above under Task 2.

Unfinished goals: Task 4 was planned for the third and final year of the grant where we would study metabolic flux in xenograph tumors generated from breast cancer cells. We have been unable to complete these studies as the metabolite pool is too low to warrant the sacrifice of animals.

Statement of work: *Task 3b*. We are in the process of analyzing the effects of carbonic anhydrase inhibition (which theoretically will neutralize the extracellular pH) on ^{13}C-glucose metabolism (this will be done during the no cost extension period).

Key Research Accomplishments:

During this second year, we have continued to work with Sue-Wei Luu an undergraduate who has just entered Medical School here at the University of Florida. Erilda Taragini played a significant role in performing the experiments using 6AN. She has now graduated and moved on. An additional undergraduate student, Gabrielle Fisher, has added to these studies with replicates to verify the pentose phosphate shunt activity. These later studies, together, demonstrated that the pentose phosphate shunt (PPS) indeed plays a significant role in glucose metabolism in the MDA-MB-231 cells. If this were not a major player, then 6-phosphogluconate, the substrate for the second dehydrogenase activity in the PPS, would not have accumulated. This (accumulation) led to the inhibition of glycolysis through the block in phosphoglucose isomerase.

Reportable Outcomes:

We are presenting our work at the ERA of Hope Symposium in August, 2011 [5]. In addition we have put together a manuscript which will be submitted within the month. This manuscript will describe the unique phenomenon in triple negative breast cancer cells which may give these cells a selective advantage over normal cells in the tumor microenvironment. In addition, the data describing the inhibition of the pentose phosphate shunt suggest that this path plays an important role in metabolic flux.

Conclusions:

We have made good progress toward our goals having completed Task 1 (and received IACUC renewal) and completed the experiments defined in Tasks 2 and 3 (with the exception of the carbonic anhydrase inhibitor studies which will be done in the no cost extension period). We have set up a uniform system that can be utilized for analysis of ^{13}C-isotopomers across multiple cell types. We have shown that hypoxia treatment of a triple negative cell type decreases flux from glycolysis through the TCA but ramps up an anapleurotic step to engage the down-regulated TCA cycle, likely to maintain ATP levels though oxidative phosphorylation. We have shown that 6AN blocks glucose metabolism suggesting that the PPS plays a significant role in glucose utilization in the MDA-MB-231 cells. Our future directions include the identification of the novel anapleurotic pool that we observed in hypoxic breast cancer cells.

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